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Morpho-pathological and genomics-based identification of the Aspergillus species infecting pomegranate with nano- driven solution

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Pomegranate (*Punica granatum* L.) is the dominant fruit for the tropical and sub-tropical area that belongs to the family Punicsceae. It has been cultivated throughout the world and drier parts of southern Asia. Good source of carbohydrates and minerals. Among post-harvest fungal pathogens attacked on pomegranate, *Aspergillus niger* is common store fungus that causes black mold disease. It mainly attacks in the flowering stage and early fruit developing during rainy seasons without any external symptoms. Disease samples were collected from different local markets of Faisalabad. Infection of fungus was done from the infected part of the pomegranate using artificial media. The fungus was identified on a morphological basis then Koch postulates were followed to confirm pathogenicity. The proved pathogen was determined by molecular characterization. DNA extraction and PCR have done of fungus. Antifungal potential of silver nanoparticles against *Aspergillus niger* were performed in three different concentration 50ppm, 100ppm and 150 ppm and compared with control The data were collected on the 3rd, 5th and 7th day of the fungal growth in which the 150ppm concentration was very affected than other concentrations.
Keywords: Pomegranate, postharvest disease *Aspergillus niger*, nanoparticles.

INTRODUCTION

Pomegranate (*Punica granatum* L.) is the dominant fruit for the tropical and sub-tropical area that belongs to the family Punicsceae. The name of pomegranate comes from Latin words which means Apple with many seeds. It is grown across the majority of the world and the drier areas of southern Asia. Malaysia, East India, and tropical Africa are three of the world's most populous countries. Afghanistan, Iraq, Iran, Egypt, Bangladesh, China, Saudi Arabia, Burma, India, Pakistan, and Burma (Morton, 1987). Pakistan ranks tenth in the world in pomegranate production, with 57.8 thousand tons produced annually on 14.9 thousand hectares (PS, 2017). Pomegranate fruits are high in carbohydrates and minerals, including calcium, iron, sulfur, and a moderate amount of pectin (Waskar, 2006). Pomegranates are recognized for their sweet flavor, juice, and nutritional value, and they may be beneficial for heart problems, cancer, and aging issues (Drogoudi *et al.*, 2012).

Postharvest infections destroy 10-30% of the entire yield of many perishable crops, especially in undeveloped countries. More than 30% of the crop is lost due to postharvest infections (Agrios, 2003). Picking, grading, packaging, storing, and exporting fruits poorly might cause them to rot. This comes as a result of the activation and development of related pathogens that became active when the structural condition of the fruits and vegetables changed (Wilson *et al.*, 2000). *Aspergillus niger* is a fungus that belongs to the genus *Aspergillus*, It is among the most common species in *Aspergillus* genus. It is a major source of food contamination and produces a disorder known as black mold on fruits (Chiotta *et al.*, 2013). Fruits that have been infected are yellow or brownish-red with some generally light reddish color. This disease is linked to split or groove, which responsible for secondary infections on fruit stored with increase of temperatures (Yehia, 2013). *Aspergillus niger* caused fruit rot of pomegranate is one of the most severe post-harvest infections, causing pomegranate growers to lose up to 94% percent of their crop (Bardas *et al.*, 2009). In Pakistan, this disease occurs in pomegranate orchards annually, causing major yield and quality losses. The disease became more serious during the rainy season, and fruit symptoms took two forms: spherical depressed spots on the pericarp only, and black rot limited to internal fruit tissues (Jamadar *et al.*, 2011).

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Without any external symptom the fungus continues to develop within the fruit just like *Alternaria* (disease of fruit rot). Colonization of fruit by *A. niger* is closely compared with bird pecking, fruit damage, Sunbury cracking, russeting and pest infestations (Yehia, 2013).

Nanoparticles (NPs) have a range of beneficial and harmful effects in plant sciences, depending on the metal, form, scale, concentration, and production method (Lee *et al.,* 2008). Nanoparticles are composed of macromolecular materials in which the active component (drug or physiologically active substance) is mixed, trapped, encapsulated, or coated and has a diameter of 1 to 100nm. (Jeevanandam *et al.,* 2018). The aim of this study is those very few work has been done in Pakistan for the postharvest disease such as black mold disease of pomegranate caused by *Aspergillus niger*, therefore, the present research has been done to determine the black mold fungi and management by silver nanoparticles is a safe management.

MATERIALS AND METHODS

Sample collection: Infected specimens Pomegranate fruits with rot symptoms were picked in Faisalabad's local markets (e.g., Ghulam Abad Manddhie, Sardar Bypass Manddhie, Junng Bazar, etc.) and also Fresh and stable pomegranates had harvested from a well-managed orchard for pathogenicity processing from Horticulture orchard in University of Agriculture Faisalabad.

***Preparation of PDA media*:** Peeled potatoes of 250g were taken and boiled in 500ml water for 20min to get the potato starch and 20g dextrose was added in 500ml flask containing water. 20g agar agar in another flask was dissolved in 300mL of water. After those contents of both flasks were combined collectively to make the volume of I litter and then autoclaved at (121°C) at 15psi for 20 minutes.

Isolation of fungal pathogen: Before being cut up into small parts, the epidermal tissues taken from the rot section of the pomegranate was disinfected with 70% ethanol (5 mm in diameter). In Potato Dextrose Agar (PDA) the culture was transferred and then incubated at 28 centigrade for 48 hours. The edges of the colony's Mycelia were collected and transferred into new PDA plates for purification. The last step repeated again, and single colonies of each pathogen was isolated for the further detection.

***Morphological Identification*:** Fungal colonies from the third generation of isolated pathogens were cultured on fresh PDA plates and incubated for 12 days at 28 °C. Each day, observed the morphological features of the colonies. An optical microscope was used to examine the characteristic of the spores and hyphae. The colony feature shows that the first white to yellow mycelial growth occurs on the surface of the culture plate with black conidia. The black conidia raised on the PDA culture some time touched the laid of the growth culture. The morphological feature were compared with previous study to identify the pathogens (Silva *et al*., 2011 and Slippers *et al*., 2004).

***Pathogenicity Test*:** The using of sterile double-distilled water, a spore suspension were used in this procedure with a concentration of 1×10^6 cells/m. A healthy pomegranate were wrapped with a sterile pipette tip (significantly 4 mm in diameter,1×1 mm, with 2 opposite sides holes in each fruit), and 20 µL of spore suspension was transferred to the injured place. A mycelial disc of similar size (5 mm in diameter) was punched from the PDA board through an inoculating needle, which placed to the wound site of the pathogen without spore formation. The control pomegranate was treated with the same method for wound treatment and inoculated with sterile double distilled water into 20 µL were injected. All injected pomegranates were preserved in a dark environment at 28°C and 90% relative humidity for 30 minutes following inoculation. Size of the lesion were measured using the crossover method on the third, seventh and tenth day after infection. Size of the lesion were measured using the crossover method on the third, seventh and tenth day after infection.

Molecular characteristics

Extraction of DNA: According to Platner *et al.,* (2009). DNA was extracted from fungal cultures. Fungal spores were inoculated on potato dextrose broth (100ml) and incubated for 72 hours at 28°C and 180rpm in a shaking incubator. The culture was then centrifuged for 25-30 minutes at 13000 rpm in a 50ml falcon tube. (Before beginning work, turn on and set the water bath to 65°C, and centrifuge to 4°C). After centrifugation, remove the supernatant and grind the pellets with sterilized sand and warmed CTAB (cetyl trimethylammonium bromide) solution in a pestle mortal. Transfer the grinded sample into 15ml falcon tube after grinding and incubated in water bath for 30 minutes at 65ºĊ, inverted the falcon after every 10-minutes. After 30 minutes, loaded each falcon tube with an equal amount of chloroform isoamyl alcohol, shake well, and centrifuge at 4°C for 15 minutes at 9000rpm. Supernatant was taken and an equivalent amount of chloroform isoamyl alcohol was added before centrifugation. After centrifugation, the supernatant was transferred to a fresh sterile falcon tube, and cooled 2 propanol was added in equal parts. The tube was then centrifuged for 10 minutes at 4°C at 9000rpm. Samples were centrifuged, a pellet produced. The supernatant was then discarded, and the pellet was rinsed in 70% ethanol before being centrifuged for 2-3 minutes. The falcon was put inverted on tissue paper until it dried after the supernatant was discarded. The pellets were then dissolved in 25µL of R40. A Nano drop spectrophotometer was used to measure DNA (Plattner *et al*., 2009).

Agarose gel electrophoresis: For PCR product analysis, electrophoresis (1 percent agarose gel) was used. The agarose gel was obtained by adding 1 gram of agarose in 100 mL of 0.5X TAE buffer and added staining agent with 2.5

microliters of ethidium bromide, Gel samples were put into gel wells after solidification, and voltage was applied to perform the experiment. The macromolecules were separated using an agarose gel based on their mass to charge ratio. Gel documentation system (GDS) was used to examine the gel and record the gel images.

***Purification of PCR product*:** A PCR purification kit was used to purification of the PCR product. The purification kit manufacturer's protocol was followed. Transfer the PCR product in eppendrof. Added 5 volume favor prep PCR cleaning (FAPC) buffer in eppendrof having PCR sample. Place FAPC column into collection tube. Vortex the mixture of FAPC column and PCR product and transfer into FAPC column (incubate the elution buffer at 55ºC). Centrifuged the mixture at 9000 rpm for 30 sec. Discard flow through. Add 600µl wash buffer and centrifuged at 9000 rpm for 30 sec. Discard flow through and centrifuged at 13000 rpm for 3 min. Shift the column in elution tube. Add 40µl elution buffer in the center of membrane of FAPC column then wait for one minute. Again, take eluted product and passed through the membrane of FAPC column and centrifuged at 13000rpm for one minute. Take flow through with pipett, again poured into column and centrifuge for 1 minute at 13000 rpm. Stored at -20ºC

***Sequencing*:** Purified PCR products were put into sequencing tubes with ITS1 primer and transported for sequencing after purification. Using software, the sequences were cut and aligned. A phylogenetic tree was constructed using aligned sequences. A phylogenetic tree was built to determine how closely related the sequences in queried are to one another. Trimmed sequences were searched against the NCBI database and literature using the BLAST program. Gen-Bank was used to store the trimmed sequences. The NCBI database was used to obtain and align ITS sequences of known isolates. After that, phylogenetic analysis was carried out.

***Evaluation of antifungal activity of silver nanoparticles*:** In vitro, purified silver nanoparticles different concentration (50ppm, 100ppm, 150ppm) were prepared. Dilute the purified silver nanoparticles. Antifungal activity of silver nanoparticles was evaluated by using poisoned food technique.

Table 1. Silver nanoparticle treatments against conform pathogen to inhibit the growth of fungal isolated.

Treatments	Concentration
T1	Silver nanoparticles (50ppm)
T2	Silver nanoparticles (100ppm)
T3	Silver nanoparticles (150ppm)
T4	Control

RESULTS

Morphological Identification: The Morphological identification have been done under the microscope. While for further microscopic identification of the *Aspergillus niger* spores used compound microscope. Morphological and Microscopic identification of *Aspergillus niegr* shown that the most common and simply identifiable *Aspergillus* species. First white to yellow mycelial growth occur on the surface of the culture plate with black conidia. The structure of a compact layer of dark brown to black conidial beads is produced, as shown in figure 1.

Figure 1. Grouth of *Aspergillus niger* in PDA plates.

The black conidia raised on the PDA culture some time touched the laid of the growth culture. Microscopic identification of *Aspergillus niegr* show that Conidia were dark brown, globose, to 3 mm in size by 15 to 20 µm in diameter, having typically radiated heads which splitting to form loose columns with age. Conidiophores were hyaline, smooth-walled or turning dark to the vesicle. Conidia are rough-walled, globose to sub-globose (3.5 to 5 µm in thickness) and dark brown to black in color as shown in Fig. 2.

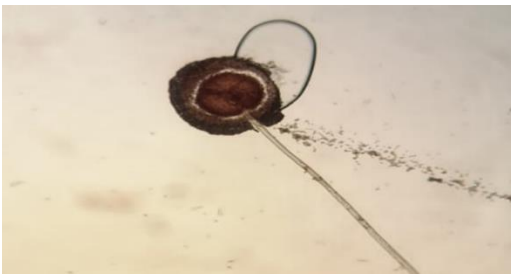

Figure 2. The microscopic structure of *Aspergillus niger*.

Pathogenicity Test: Koch's postulates were followed to confirm the pathogenicity of isolated fungus from infected fruits. Aspergillus spp was proved as a pathogen.

***I. solation of the fungal culture from the host*:**

In the first stipe of the pathogenicity test, the fungal culture isolated from the infected fruits after processing the fungus colony showed similar morphology, which identified earlier that white to yellow mycelial growth occur on the culture plate surface, which had black conidia. On PDA medium, colonics comprise a dense white or yellow mycelial growth with a compact compact layer of dark brown to black conidial beads structure produced on the head of the conidiophores.

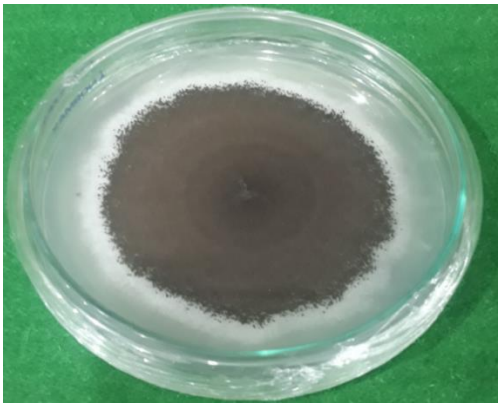

Figure 3. Isolated culture of *Aspergillus niger*.

II. Inoculation of the fungal culture to the healthy host fruits for symptoms development and compared with disease fruits: The second stipe of the pathogenicity test on the fruit surface changed the fruits' structural condition. Fruit is generally light reddish color, as shown in figure number 4. The inoculated sample shown off color on the fruit surface and black mold rotting was developed. The diseased symptoms samples were compared with collected disease specimens brought from the market as the pathogen infected the fruit. For further confirmation, the pathogen re-isolates of fungus in culture plates.

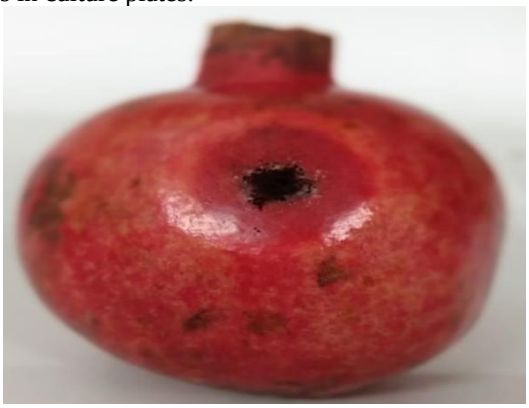

Figure 4. Isolated fungus on healthy Pomegranate.

Re-isolation of the pathogen from inoculated fruits: After the tested fruits were processed and transferred to culture on PDA plates, the same colony structure was observed in the first pathogenicity stipe. The colony mycelial is white, yellow shaped, and has black conidia. The compact layer of dark brown to black conidial beads and conidiophores were hyaline, smooth-walled, or turning dark to the vesicle structure produced, as shown in figure 5. The culture of the fungus compared with the growth culture, which is already discussed in the pathogen's morphological structure as shown in figure 1, the growth of *Aspergillus niger* on PDA plates. As a result, the pathogen was confirmed by the colony of fungus.

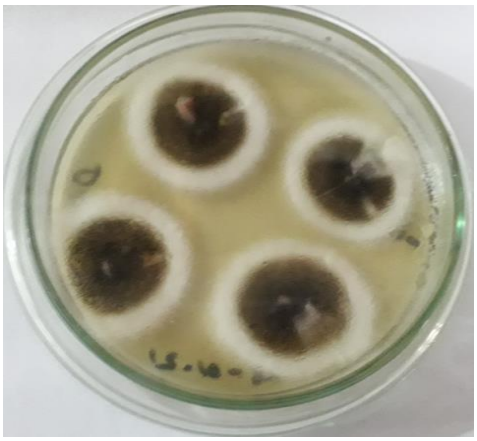

Figure 5. Re-isolated of *Aspergillus niger*.

Morphological identification of the pathogen

Morphological identification of the pathogen and compared the microscopic characters with previous morphological study of fungus. It is the last stipe of Koch's postulate. The morphological characterization the conidia have dark brown to black. Conidia were globose, to 3 mm in size by 15 to 20 µm in diameter, having typically radiated heads which splitting to form loose columns with age. Conidiophores were hyaline, smooth-walled or turning dark to the vesicle. The morphological characters compare with figure 2 which explained already in the morphology of the fungus.

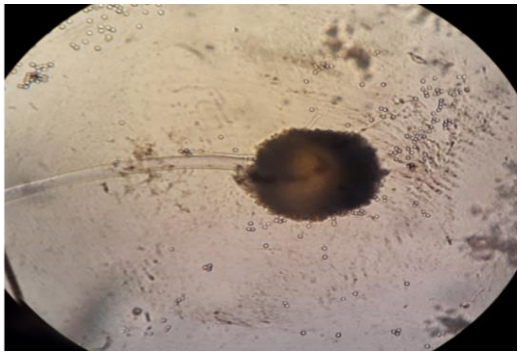

Figure 6. Microscopic structure of *Aspergillus niger*.

In vitro evaluation of nano particle against isolated fungus: The prepared silver nanoparticle used for statistical analysis in the three different concentration such as 50ppm, 100ppm and 150ppm and the data were collected after 3rd, 5th and 7th day of isolated fungus.

Table 2. LSD All-pairwise comparison test with different concentration of silver nanoparticles on the mycelial growth of *Aspergillus niger* after 3 days.

Concentration	Means	Homogeneous groups
Control (0ppm)	3.550	A
50ppm	3.025	B
100ppm	2.725	C
150ppm	2.450	D

Alpha = 0.05 Standard Error for Comparison = 0.0854 Critical T Value = 2.179. Critical Value for Comparison = 0.1861. All 4 means are significantly different from one another. The data in table 2 shows the effect of different concentrations of nanoparticles after three days on the mycelial growth of *Aspergillus niger*. The concentration of 150ppm was founded most effective in reducing the mycelial growth (2.4500 cm), followed by a concentration of 100ppm with a mean value of (2.7250 cm). At the same time, Whereas concentration of 50ppm least effective controlling mycelial growth of *A, niger* with the mean value of (3.0250 cm).

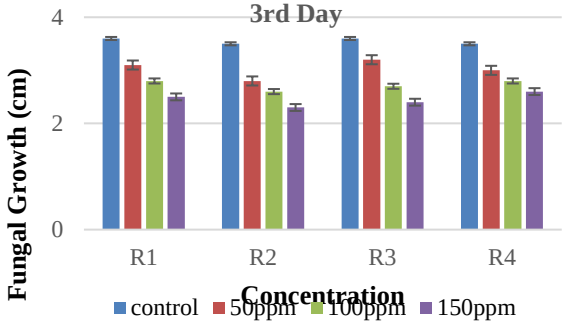

Figure 7. Effect of nanoparticles at different concentrations on the mycelial growth of *Aspergillus niger* after 3 days.

Table 3. LSD All-pairwise comparison test with different concentration of silver nanoparticles on the mycelial growth of *Aspergillus niger* after 5 days.

Concentration	Means	Homogeneous groups
Control (0ppm)	4.000	A
50ppm	3.325	B
100ppm	2.900	C
150ppm	2.575	D

Alpha = 0.05 Standard Error for Comparison = 0.0854 Critical T Value = 2.179
Critical Value for Comparison = 0.1861 All 4 means are significantly different from one another.

The data presented in table 4 shows the effect of different concentration of nanoparticles after 5 days on the mycelial growth of *Aspergillus niger*. The concentration of 150ppm was founded most effective in reducing the mycelial growth (2.5750 cm), followed by concentration of 100ppm with the mean value of (2.9000 cm). Whereas concentration of 50ppm least effective controlling mycelial growth of *A, niger* with the mean value of (3.3250 cm)

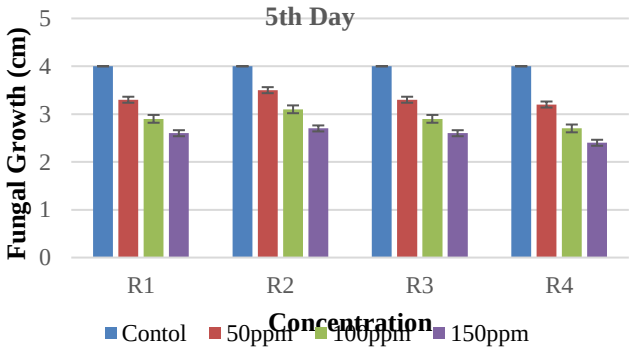

Figure 8. Effect of nanoparticles at different concentrations on the mycelial growth of *Aspergillus niger* after 5 days.

Table 4. LSD All-pairwise comparison test with different concentration of silver nanoparticles on the mycelial growth of *Aspergillus* niger after 7 days

Concentration	Means	Homogeneous groups
Control (0ppm)	4.300	A
50ppm	3.650	B
100ppm	3.200	C
150ppm	2.875	D

Alpha = 0.05 Standard Error for Comparison = 0.0810 Critical T Value = 2.179
Critical Value for Comparison = 0.1765All 4 means are significantly different from one another.

The data presented in table 4.5 shows the effect of different concentration of nanoparticles after 7 days on the mycelial growth of *Aspergillus niger*. The concentration of 150ppm was founded most effective in reducing the mycelial growth (2.8750 cm), followed by concentration of 100ppm with the mean value of (3.2000 cm). Whereas concentration of 50ppm least effective controlling mycelial growth of *A, niger* with the mean value of (3.6500 cm).

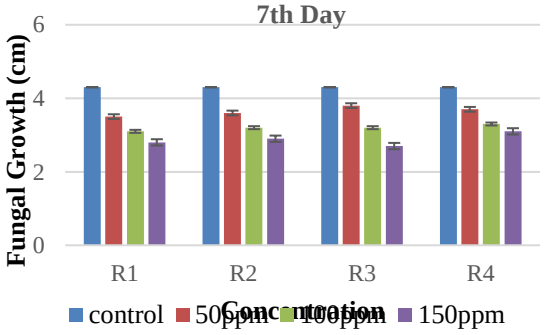

Figure 9. Effect of nanoparticles at different concentrations on the mycelial growth of *Aspergillus niger* after 7 days.

DISCUSSION

This research also relate to the application of nanotechnology in plant pathology is still in the early stages, For example, nano-fungicides, nano-pesticides and nano-herbicides are being used extensively in agriculture practices (Prasad *et al*., 2014). We used this emerging technology for the black mold postharvest disease caused by Aspergillus niger in pomegranate, which is very common in Pakistan. As for many results, different concentrations (50ppm. 100ppm. 150ppm) of silver nanoparticles were used to manage black mold diseased caused by *Aspergillus niger* in pomegranate fruit but 150ppm concentration was most effective in controlling the disease as compared to other concentrations. Instead of others, 150ppm concentration growth of inhibition was grater. Thakkar *et al*., 2010 explained that work, the control methods like use of chemicals or very costly, Microbial synthesis of nanoparticles is environment friendly and natural method, other nanoparticles like phosphorus (Gurunathan *et al*., 2014). Copper and silver based nanoparticles (Ali *et al*., 2014) were also evaluated, but silver nanoparticles show the highest efficacy against different diseases caused by fungi. The silver nanoparticles cause great reduction in fungal growth, mycelia and their hyphae effectively without any hazardous effect (Herrera and Martínez , 2001; Zhao and Stevens, 1998). So, on this silver nanoparticles were used against pathogenic fungi.

Recent studies prove that the nanotechnology have wide application in controlling plant disease (Bhattacharyya *et al*., 2011, Rai and Ingle, 2012). The technique is becoming popular among the Farmers that can prevent diseases. Tormey *et al*. (2007) describe that The insertion of the resistant gene can reduce the use of fungicides in plants; not only silver nanoparticles but aims the metallic nanoparticles prove to have the antimicrobial activity, Different scientist evaluate the antimicrobial potential of nanoparticles against various pathogens like Oh *et al*., 2006 evaluate the antimicrobial potential of nanoparticles against *Botrytis cineria*, Min *et al*., 2009 assessed against seclrotium, Kasprowiez *et al*., 2010 and Musarrat *et al*., 2010 evaluated against the Fusarium spp, Fateixa *et al*., 2009 tested for the *Aspergillus niger*, tested for different fungal pathogens like *Alternaria ulternata, Seclerotinia sclerotium, Curvularia, Macrophomina phaseolina, Botrytis cineria,* and *lunata*. Many other scientists also proved that the silver nanoparticles is also effective for the control of canker in guava caused by the *Neopestaliopais sp*

***Conclusion*:** On the basis of the present research, the postharvest fungus most probably caused by *Aspergillus niger* based on morphological identification and molecular characterization and concluded that high concentration of the nano-particles (such as 150ppm) were inhibited the growth of *Aspergillus niger* on the culture plates

***Conflict of interest*:** Author has no conflict of interest.

***Authors Contributions statement*:** Suleman Khan and B. Khalid wrote and Nurettin Baran edited the manuscript and did the whole research in Fungal Molecular Biology (FMB) laboratory.

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***Ethical statement*:** This article does not contain any studies with human participants or animal performed by any of the authors.

***Availability of data and material*:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

Code Availability: Not applicable

***Consent to participate*:** Suleman Khan wrote and edited the manuscript and did the whole research in Fungal Molecular Biology (FMB) laboratory

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***Consent for publication*:** All authors are giving the consent to publish this research article in Phytopathogenomics and Disease Control

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